Enhancement of the Gelling Properties of Sardine Surimi with Transglutaminase and Optimization of its Activity Using Response Surface Methodology

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Abstract

In order to improve the gelling properties of sardine surimi gel and to determine the maximum activity range of microbial transglutaminase (MTGase), we investigated the characteristics of surimi gel as a function of MTGase concentration, as well as temperature and reaction time, using the response surface methodology. Specifically, we assessed the following mechanical and physicochemical characteristics of the gel: rheological properties, disulphide bond and total sulphydryl group content, and water-holding capacity. Our results demonstrated that temperature and enzyme concentration had more influence than reaction time on all dependent variables, incorporation of MTGase markedly ameliorated all the responses. The optimal properties were predicted to be obtained by employing the optimised setting conditions as follows: a concentration of MTGase of 10 g/kg of surimi at 45°C for 1 h. All the mathematical models derived for the various responses were found to be a good fit to predict the data.

Keywords: Microbial transglutaminase; Setting; Sardine surimi; Gelling; Response surface methodology

Introduction

In recent years, the increasing demand for "fresh food products" worldwide has prompted attempts to better understand and study restructured product. The addition of ingredients or additives can lead to improve these products in order to make them with new textual properties and/or a new appearance. Most of these products, particularly seafood analogues, are made from surimi of different sources of protein. Surimi is a structure of myofibrillar protein concentrates obtained from fish muscle, with a high commercial value and a wide range of applications in seafood production [1]. Given the limited availability of white muscle fish due to their overexploitation, it would be desirable to use lower-quality surimi from other species and to improve it by further processing [2]. Therefore, more attention has been paid to dark muscle fish species as an alternative raw material [3-5].

Among all dark-fleshed fish species, sardine was one of the most abundant species caught in Tunisia. The use of this small pelagic fish for surimi production is a promising way and an alternative to revalue and convert underutilized fish protein resources into foods of fish for surimi production is a promising way and alternative to revalue and convert underutilized fish protein resources into foods of premium quality, particularly protein gel-based products. However, the characteristics of surimi gel depend on the properties of the myofibrillar proteins, and these are determined by the species and freshness of the fish, as well as the processing parameters [6].

Small pelagic fish species, such as sardine, produce surimi with poor gel characteristics due to the high content of dark muscle, comprising a considerable amount of lipids and sarcoplasmic proteins [7,8]. The presence of sarcoplasmic proteins of dark muscle has often been cited as one of the reasons for the poorer gel properties of dark muscle fish compared to light muscle fish [9]. In this context, microbial transglutaminase (MTGase) has been studied as a means of improving the textural characteristics and mechanical properties of fish as well as meat products [10]. MTGase induces the formation of ε-(γ-glutamyl) lysine cross-link in the proteins via acyl transfer between the ε-amino groups of a lysine residue and γ-amide group of a glutamine residue [11]. The reactions promoted by the enzyme create profound changes in the proteins in food matrices, which results in the improvement of textural properties and stability in terms of temperature, syneresis, emulsifying properties, gelation and water-holding capacity (WHC), without changing the pH, colour, flavour or nutritional quality of food, and may even render it more nutritious due to the possibility of adding essential amino acids [12]. However, the efficiency of MTGase in improving gelation properties of proteins depends on many factors, including the amount of MTGase, type of fish, and fat content [13-15].

In addition to the gel enhancer used, setting response is an important step in the formation of surimi gel that contributes to the different gelling properties. Setting or suwari is a treatment prior to cooking, which involves pre-incubation of salted surimi paste, generally at temperatures between 0°C and 40°C [16]. This results in the formation of a myosin network through cross-linking induced by endogenous transglutaminase (TCase) [17,18]. Considering this, a study to determine the optimal conditions for setting, using MTGase, can be based on analysing the relationship between enzyme concentration, time and temperature.

To determine the best conditions for TGase activity in foods, several authors have used the response surface methodology (RSM) [19-21]. This methodology develops a suitable experimental design by collecting statistical and mathematical tools that are useful for the modelling and analysis of problems in which a response of interest is influenced by several variables, with the objective of optimizing the response [22]. The RSM involves full factorial research to explore

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simultaneous, systematic and efficient variations in the important components, identifying potential interactions and higher-order effects, and thereby determining the optimal operational conditions [23]. Although MTGase has been successfully used for improving the gelation properties of surimi, no information has been reported regarding the use of MTGase in surimi gel from dark-fleshed fish species, such as sardine. The aim of this study was to use sardine (Sarda pilchardus) resource and transform it from low-valued fish to high-value-added product "surimi gel", to evaluate the effects of MTGase in sardine surimi gel and to optimize the conditions for enzyme activity during setting (concentration, temperature and time) using the RSM.

Materials and Methods

Microbial transglaminase

MTGase from Streptovercillium mobaraense was supplied by Ajinomoto USA, INC. (Teanack, NJ, USA). Enzyme activity reported by the supplier was 100 units/g dry weights. The enzyme powder consisted of 99% maltodextrin and 1% enzyme by mass.

Surimi paste preparation

In order to prepare surimi by the conventional washing process [24], fresh sardine (Sarda pilchardus) was purchased from a fish market in Tunisia. Fish were headed, eviscerated and washed. Skin and bones were removed by hand in the laboratory. Fillets were minced in a meat mincer (TC-32 EL/80 Tre Spade, Torino, Italy). Fish mince was washed with cold water (4°C) using a water-to-mince ratio of 3:1 (v/w). The mixture was stirred gently for 10 min and the washed mince was filtered with a layer of nylon screen. This washing process was performed thrice. Finally, the washed mince was dewatered by centrifugation at a speed of 700×g for 15 min at 4°C (Model CE 21 K, Grandimpianti, Belluno, Italy). A cryoprotectant mixture was added to the washed mince (4% sorbitol and 4% sucrose w/w). Surimi was packed into polyethylene bags (250 g) and stored at -20°C until used for gel preparation. The storage time did not exceed 1 month.

Surimi gel preparation

Frozen surimi samples were partially thawed at 4°C, then cut into small pieces and chopped with 2.5% NaCl (w/w) for 3 min. MTGase was then added to the surimi paste and the mixture was chopped for another 5 min. Then moisture content of the surimi was adjusted to 80% with iced water and the chopping was continued for 15 min. For the entire chopping process, the temperature was kept below 10°C.

The paste was stuffed into stainless steel tubes (diameter=2.5 cm; length=15 cm), inner wall of which was coated with a film of vegetable oil to prevent gel adhesion. Both ends of the tubes were sealed tightly. The paste samples were allowed to set and then heated at 90°C for 20 min. The gel samples were cooled rapidly in iced water and kept at 4°C overnight until analysis.

Rheological measurements

Linear dynamic viscoelasticity measurements were determined using a controlled-stress rheometer (AR2000) from TA Instruments (New Castle, DE, USA) equipped with two parallel plates (40 mm diameter) with rough surfaces, setting a gap between plates of 0.5 mm [25]. Before measurement, the gels were tempered at room temperature and cut to size, namely, to the same diameter as that of the plate and at a thickness of 0.5 mm. Samples were covered with a thin film of petroleum jelly to avoid evaporation. All determinations were carried out at least in triplicate.

Stress sweep tests: In order to determine the linear viscoelastic (LVE) region, stress sweeps were conducted at 6.28 rad/s with a constant temperature at 25°C. The shear stress (σ) of the input signal was programmed to range from 1 to 1000 Pa.

Frequency sweep tests: Frequency sweep tests were carried out at different frequencies ranging from 10 to 0.1 Hz under a constant shear strain amplitude (γ=0.2%) within the LVE range. Storage modulus (G’), loss modulus (G”) and loss tangent (tan δ) were recorded at 1 Hz.

Disulphide bonds

The disulphide bond (DB) content was determined using a 2-nitro-5-thiosulphobenzoate assay method [26]. Freshly prepared 2-nitro-5-thiosulphobenzoate assay solution (3 ml) was added to the protein solution (0.5 ml) and the mixture was allowed to set in the dark at room temperature (26°C to 28°C) for 25 min. Absorbance was measured at 412 nm. DB content was calculated using the molar extinction coefficient of 13,900 M⁻¹ cm⁻¹ using a Genesys-20 spectrophotometer (Thermo Scientific, USA).

Total sulphhydryl group content

The total sulphhydryl (TSH) content was determined using 5,5'-dithio-bis (2-nitrobenzoic acid) method [27] with some modifications. Samples (70 μL of protein solution) were homogenized with 1 ml of solubilizing buffer (20 mMTris–HCl, 8 M urea, 10 mM EDTA, pH 8.0). An aliquot (100 μL) of Ellman’s reagent was added. The mixture was incubated in the dark at room temperature for 30 min. As for DB content, the amount of TSH was measured at 412 nm with a molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ using a Genesys-20 spectrophotometer (Thermo Scientific, USA).

Water-holding capacity

WHC was evaluated by a centrifuge method [28]. It was expressed as the percentage of the initial water remaining in the sample after centrifugation. Each value is the mean (standard deviation) of at least four measurements.

Response surface methodology

In order to determine the best conditions for the production of sardine surimi gel, Response surface methodology (RSM) was applied to derive mathematical models to estimate the effects design and the interactions of the independent variables: enzyme concentration (x₁), temperature (x₂) and incubation time (x₃) on the storage modulus (G’), loss modulus (G”) y, disulphide bond content (DB y), total sulphhydryl group content (TSH y) and water-holding capacity (WHC y) of surimi gel samples which were the selected responses for this research.

The experimental design followed a second-order factorial structure [29] with 4 replications at the centre point to estimate the experimental error, leading to 30 experiments, carried out in a random order and in triplicate.

Table 1 presents the variables used in the optimization of the setting process during sardine surimi production including fixed variables at usual values.

In order to estimate the response, an empirical model was constructed based on a second-order polynomial (1):
The results and discussion show that enzyme concentration did not significantly affect the disulfide bond (y3) and total sulfhydryl group content (y4). The analysis of the regression coefficients and the main experimental trends are presented in Table 3. It can be concluded that temperature and enzyme concentration have the most influence on G′, followed by the interaction between temperature and time. Using the models, it is possible to predict the results and plot them as a response surface in three-dimensional graphs. The response surface for G′ (y1) is illustrated in the Figure 1 which describes the dependence of G′ on enzyme concentration and temperature at the different incubation times used. From the plot, it can be seen that increasing enzyme concentration and temperature allow us to reach higher values of G′ at shorter times, but at longer times, high enzyme concentration and temperature lead to a decrease in y1.

For loss modulus G″ (y2), a similar behaviour was observed, the experimental results varied within the wide range of 1845 to 13897 Pa (Table 2). Analysis of the regression coefficients and the main experimental trends suggest the same results as G′ (y1) (Table 3). Temperature and enzyme concentration have the most influence on G″, followed by the interaction between temperature and time. The resulting variation pattern is shown in Figure 2 where MTGase concentration and temperature were the variables that most influenced the setting of sardine surimi gel.

### Disulfide bond and total sulfhydryl group content

DB and TSH content were also used as responses because they are major parameters for evaluating the gelling properties of surimi. The presence of sulfhydryl groups in surimi is necessary for gel strengthening. High temperatures during heating led to further oxidation of sulfhydryl groups with a subsequent disulfide bond formation [44].

DB (y2) and TSH (y3) showed behaviour different to y1 and y2. The experimental results varied within the narrow ranges of 23.99% to 33.6% and 14.71 to 1.14 mmol/g proteins for DB and TSH, respectively. The most favourable conditions were defined by the maximum value of enzyme concentration, the highest value of temperature and the shortest time (experiment 28). Analysis of the regression coefficients for y3 and y4 showed that enzyme concentration did not significantly affect DB content. The variation of DB and TSH contents with

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### Results and Discussion

#### Parameter ranges studied

The main effects and the interactions of MTGase concentration, temperature and reaction time during setting of sardine surimi were assessed using the RSM. Many researchers apply RSM as a mathematical modelling tool in bioprocess optimization [30,31]. By using RSM, process variables could be controlled together to result in maximum product properties with desired characteristics [32,33].

Setting can be performed at low (0°C to 4°C), medium (25°C) or high (40°C) temperatures [34], and the choice of temperature may lead to different gelling properties, since gel setting behaviour is fish species and temperature dependent. At low temperature, setting takes longer, and so it is not commonly implemented in industry. So far, high-temperature setting is more widely used in Thailand, given the shorter time required, but there is a risk of protein degradation, due to modori-inducing proteinase, which is generally active at 50 to 60°C [35]. Therefore, a medium-temperature setting may be considered the best option for manufacturers to obtain the highest gel quality with negligible proteolysis.

In line with this, we studied temperatures between 25°C and 45°C, because 25°C is the temperature commonly recommended for setting in cold water species such as Alaska Pollock (Theragra chalcogramma) [36], while 45°C is the recommended maximum, to avoid the modori phenomenon in the range 50°C to 70°C [37]. Regarding reaction time, we collected data between 1 h and 5 h, because it has been reported that MTGase is active for between 2 h and 5 h in Alaska pollock at 25°C [38].

The range for the variable enzyme concentration (0 g/kg -10 g/kg) was chosen according to the studies which deal with the improvement of the mechanical properties of surimi produced from striped mullet (Mugil cephalus) and silver carp (Hypophthalmichthys molitrix) respectively [39,40].

#### Dynamic viscoelasticity measurements

The storage and loss modulus (G′ and G″) were considered as responses, since research on surimi rheological properties has shown the importance of the viscoelastic moduli G′ and G″ in the nominal quality of surimi [41]. Moreover, the use of MTGase, through the cross-links it promotes, enables highly elastic and irreversible gels to be obtained in different substrates, even at relatively low protein concentrations [42,43].

Table 2 summarizes the operational conditions tested as well as the experimental and predicted response values determined for the dependent variables obtained from this experimental design. It can be observed that the experimental values for storage modulus G′ (y1) varied over a wide range (3121 Pa-63520 Pa).

The analysis of the regression coefficients and the main experimental trends are presented in Table 3. It can be concluded that temperature and enzyme concentration have the most influence on G′, followed by the interaction between temperature and time. Using the models, it is possible to predict the results and plot them as a response surface in three-dimensional graphs. The response surface for G′ (y1) is illustrated in the Figure 1 which describes the dependence of G′ on enzyme concentration and temperature at the different incubation times used. From the plot, it can be seen that increasing enzyme concentration and temperature allow us to reach higher values of G′ at shorter times, but at longer times, high enzyme concentration and temperature lead to a decrease in y1.

For loss modulus G″ (y2), a similar behaviour was observed, the experimental results varied within the wide range of 1845 to 13897 Pa (Table 2). Analysis of the regression coefficients and the main experimental trends suggest the same results as G′ (y1) (Table 3). Temperature and enzyme concentration have the most influence on G″, followed by the interaction between temperature and time. The resulting variation pattern is shown in Figure 2 where MTGase concentration and temperature were the variables that most influenced the setting of sardine surimi gel.

### Table 1: Variables used for the experimental design.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Unit</th>
<th>Nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme concentration</td>
<td>g/Kg</td>
<td>x1</td>
</tr>
<tr>
<td>Temperature</td>
<td>°C</td>
<td>x2</td>
</tr>
<tr>
<td>Incubation time</td>
<td>h</td>
<td>x3</td>
</tr>
<tr>
<td>G′</td>
<td>Pa</td>
<td>y1</td>
</tr>
<tr>
<td>G″</td>
<td>Pa</td>
<td>y2</td>
</tr>
<tr>
<td>Disulphide bonds</td>
<td>%</td>
<td>y3</td>
</tr>
<tr>
<td>Total sulfhydryl group</td>
<td>mmol/g proteins</td>
<td>y4</td>
</tr>
<tr>
<td>Water holding capacity</td>
<td>%</td>
<td>y5</td>
</tr>
</tbody>
</table>

The regression model is expressed as:

\[
y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 (1)
\]

Where, \(y\) is the estimated response, \(\beta_0\) the model constant, \(\beta_i\) the coefficients of the linear term, \(\beta_{ij}\) the coefficients of the quadratic term, \(\beta_{ij}\) the coefficients of interaction between the factors, \(x_i\) and \(x_j\) the design variables, \(k\) the number of factors, and \(i\) and \(j\) the coded factors of the system. The experiments were assessed using the Design-Expert software (Statease Inc., Minneapolis, USA, version 7.0). For each response, the coefficients were calculated by regression analysis and the significance of the derived model equation was determined using the analysis of variance (ANOVA).
enzyme concentration and temperature at several incubation times are presented in Figures 3 and 4 respectively. Notably, using a long incubation time did not induce oxidation of sulfhydryl groups and make it possible to reach higher values of $y_5$. In Figure 5, it can be observed that the model predicts concentration, the highest value of temperature and the shortest time favourable conditions were defined by the maximum value of enzyme concentration, the greater the number of inter- and intra-chain peptide cross-links and the weaker the protein–water interaction. Thus, the amount of enzyme added should be carefully evaluated because, in appropriate concentrations, MTGase yields stable gels with relatively high porosity that are able to immobilize water more efficiently. As a result of this increase in WHC, protein gels obtained have better textural properties, in terms of bond strength, stiffness, cohesion, chewability and elasticity [45,46].

The experimental results of WHC varied within the narrow range of 49.25% to 62.85%. Analysis of the main experimental trends and the analysis of the main experimental trends and the interaction between temperature and incubation time. The most favourable conditions were defined by the maximum value of enzyme concentration, the highest value of temperature and the shortest time (experiment 28). In Figure 5, it can be observed that the model predicts that an increase in temperature and enzyme concentration would make it possible to reach higher values of $y_5$ at short times, but that at long times, high temperatures lead to a decrease in WHC, protein gels obtained have better textural properties, in terms of bond strength, stiffness, cohesion, chewability and elasticity [45,46].

The last dependent variable considered as a response variable in this study was WHC, that is, the protein’s ability to take up water and retain it within a protein matrix, e.g., beef or fish muscle or a protein gel, through protein–water interactions. Hence, WHC is closely linked to gelation, emulsification and foaming properties [42]. In myofibrillar proteins, WHC is inversely correlated with enzyme concentration beyond a certain threshold concentration, because the higher the enzyme concentration, the greater the number of inter- and intra-chain peptide cross-links and the weaker the protein–water interaction. Thus, the amount of enzyme added should be carefully evaluated because, in appropriate concentrations, MTGase yields stable gels with relatively high porosity that are able to immobilize water more efficiently. As a result of this increase in WHC, protein gels obtained have better textural properties, in terms of bond strength, stiffness, cohesion, chewability and elasticity [45,46].

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Table 3: Regression co-efficients for the response surface models in terms of coded units.

<table>
<thead>
<tr>
<th>Co-efficients</th>
<th>Response</th>
<th>$y_1$</th>
<th>$y_2$</th>
<th>$y_3$</th>
<th>$y_4$</th>
<th>$y_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_0$</td>
<td>8902.69</td>
<td>3404.38</td>
<td>30.1</td>
<td>7.26</td>
<td>54.37</td>
<td></td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>6708.44</td>
<td>1484.56</td>
<td>0.33</td>
<td>-117.8</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>8652.28</td>
<td>2250.33</td>
<td>1.59</td>
<td>-2.2</td>
<td>2.42</td>
<td></td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>-814.61</td>
<td>-279.33</td>
<td>0.56</td>
<td>-0.22</td>
<td>-0.28</td>
<td></td>
</tr>
<tr>
<td>$\beta_4$</td>
<td>558.56</td>
<td>669.46</td>
<td>-0.19</td>
<td>0.56</td>
<td>-0.79</td>
<td></td>
</tr>
<tr>
<td>$\beta_5$</td>
<td>13259.06</td>
<td>2760.79</td>
<td>-1.11</td>
<td>0.49</td>
<td>-0.38</td>
<td></td>
</tr>
<tr>
<td>$\beta_6$</td>
<td>9986.4</td>
<td>2059.13</td>
<td>0.28</td>
<td>-0.74</td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>$\beta_7$</td>
<td>781.42</td>
<td>-379.2</td>
<td>0.29</td>
<td>0.038</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>$\beta_8$</td>
<td>2258.17</td>
<td>775.75</td>
<td>0.073</td>
<td>0.26</td>
<td>-0.6</td>
<td></td>
</tr>
<tr>
<td>$\beta_9$</td>
<td>-11399.82</td>
<td>-2298.08</td>
<td>-1.66</td>
<td>2.16</td>
<td>-2.07</td>
<td></td>
</tr>
</tbody>
</table>
After checking the normality of the data using a normal probability plot of the residuals and the difference between the observed values and those predicted from the regression, we found that the experimental points were normally distributed around the line, indicating that the normality assumption was satisfied.

ANOVA and regression analysis were used to examine the statistical significance of the terms and to check the model adequacy (Table 4).

It was suggested that for a good fitted model, the coefficient of determination \( R^2 \) should not be less than 80%. The lower values of \( R^2 \) show the inappropriateness of the model to explain the relation between variables [48].
The $R^2$ values for the regression model predicting the storage modulus $G'$, the loss modulus $G''$, DB content, TSH content and WHC were found to be 0.8824, 0.9015, 0.7845, 0.9057 and 0.8671 respectively, indicating a good suitability between the observed and predicted response values.

The lack of fit is an indication of the failure of a model representing the experimental data at which points were not included in the regression or variations in the models cannot be accounted for random error [49]. When a lack of fit is significant, the response predicted is discarded.

Data indicated the insignificance of the lack of fit of all dependent variables ($F_{cal} < F_{tab}$) confirming that the quadratic model was valid for this study (Table 4).

Moreover, to validate the model, the plots of the residual versus the predicted values should present residuals scattered randomly around zero and did not reveal any outliers.

Our results (plots not shown), showed that all the values lie within the accepted range (-3 and +3) [23] for all the response variables. Thus, all the model assumptions being satisfied, the variance analysis indicated that the model was valid.

When analysing the parity plots (plots not shown) showing the distribution of predicted values versus the actual ones of the storage modulus $G'$, the loss modulus $G''$, DB content, TSH content and WHC, it can be seen a satisfactory correlation between the experimental and predictive values since the points are clustered around the diagonal line which indicates a good agreement between the polynomial regression model and the experimental results.

**Optimization of conditions using the response surface methodology**

Based on the results, it was observed that the optimum region of enzyme activity of MTGase was obtained for an MTGase concentration of 10 g/kg with 1h of incubation at 45°C (Table 5). This means that the highest degree of fish protein cross-linking occurring during sardine surimi gel production was obtained under these conditions. From the models, it can be concluded that the optimal temperature for MTGase (40°C to 45°C) is higher than that for endogenous TGase (35°C to 40°C) extracted from sardine muscle, established in a preliminary study (data not shown), and is close to the optimal temperature (50°C) for the catalysis of MTGase [50].

Finally, in order to compare the behaviour of the setting phenomenon with the addition of MTGase, Table 6 shows the results of related studies of surimi gels from different species. The optimal setting temperature and enzyme concentration for sardine were both higher than those reported for the other fish species. This could be explained...
by the optimal temperature for setting being related to fish habitat temperature [51] and to the thermal stability of myosin in each fish species [52]. Furthermore, the cross-linking reaction induced by T-Gase occurs when protein molecules and the enzyme become associated in a highly-oriented and conformation-dependent fashion during the catalytic process [53]. Therefore, optimal enzyme concentrations and setting temperatures should be determined for each type of surimi to obtain the best gel quality.

**Conclusion**

In conclusion, the application of the RSM is a practical and effective tool in optimizing the parameters of transglutaminase activity in sardine surimi gel during setting.

The enzymatic treatment considerably ameliorated the gelling properties of the surimi gel obtained, due to the high-molecular-weight polymers formed during the cross-linking reaction induced by T-Gase.

Results suggest that setting is dependent on enzymatic activity and protein denaturation/aggregation, both processes occurring during the setting phenomenon. Hence, MT-Gase can be considered a useful additive for the production of surimi gels from sardine. Sar-dine surimi gel was predicted to have optimal properties for setting under the following conditions: 45°C/1 h using 10 g of microbial T-Gase/kg of surimi. All the mathematical models derived from the various responses were found to be a good fit to predict the data.

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